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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of: Heinrich Wieland et al.

Title: PROCEDURE AND DIAGNOSTIC PRODUCT FOR THE DETERMINATION OF TRIGLYCERIDE CONTAINED IN LIPOPROTEIN

Attorney Docket No.: 1378.001US1

PATENT APPLICATION TRANSMITTAL

BOX PATENT APPLICATION
Commissioner for Patents
Washington, D.C. 20231

We are transmitting herewith the following attached items and information (as indicated with an "X"):

X Return postcard.
X **CONTINUATION** of prior International Patent Application No. PCT/EP98/08253 (under 37 CFR § 1.53(b)) comprising:
  X Specification (15 pgs, including claims numbered 1 through 34 and a 1 page Abstract).
  X Unsigned Combined Declaration and Power of Attorney (3 pgs).
X Preliminary Amendment (2 pgs).

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P.O. Box 2938, Minneapolis, MN 55402 (612-373-6900)

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By: Ann M. McCrackin
Atty: Ann M. McCrackin
Reg. No. 42,858

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S/N Unknown **PATENT**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Heinrich Wieland et al.	Examiner: Unknown
Serial No.:	Unknown	Group Art Unit: Unknown
Filed:	Herewith	Docket: 1378.001US1
Title:	PROCEDURE AND DIAGNOSTIC PRODUCT FOR THE DETERMINATION OF TRIGLYCERIDE CONTAINED IN LIPOPROTEIN	

PRELIMINARY AMENDMENT

BOX PATENT APPLICATION
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Dear Sir:

Prior to examination of the above-identified patent application, please amend as follows:

IN THE SPECIFICATION

On the first page, after the title, please insert:

--Cross-Reference to related Applications

This application is a continuation of International Patent Application No. PCT/EP98/08253, filed on December 16, 1998, which in turn is an international filing of German Patent Application No. 19756255.8 filed on December 17, 1997, all of which are incorporated herein by reference.--

IN THE CLAIMS

Claim 3, line 1, please delete "or 2".

Claim 4, line 1, please delete "one of the preceding claims" and insert --Claim 1--.

Claim 7, line 1, please delete "one of the preceding claims" and insert --Claim 1-- therefor.

Claim 8, line 1, please delete "one of the preceding claims" and insert --Claim 1-- therefor.

Claim 11, line 1, please delete "one of Claims 8 to 10" and insert --Claim 8-- therefor.

Claim 12, line 1, please delete "one of Claims 8 to 11" and insert --Claim 8-- therefor.

Claim 13, line 1, please delete "one of Claims 8 to 12" and insert --Claim 8-- therefor.

Claim 14, line 1, please delete "one of the preceding claims" and insert --Claim 1-- therefor.

Claim 16, line 1, please delete "or 15".

Claim 20, line 1, please delete "or 19".

Claim 23, line 1, please delete "one of Claims 18 to 22" and insert --Claim 18-- therefor.

Claim 24, line 1, please delete "one of Claims 18 to 23" and insert --Claim 18-- therefor.

Claim 27, line 1, please delete "one of Claims 24 to 26" and insert --Claim 24-- therefor.

Claim 28, line 1, please delete "one of Claims 24 to 27" and insert --Claim 24-- therefor.

Claim 29, line 1, please delete "one of Claims 24 to 28" and insert --Claim 24-- therefor.

Claim 30, line 1, please delete "one of Claims 18 to 29" and insert --Claim 18-- therefor.

Claim 32, line 1, please delete "or 31".

34. (CANCLED)

35. (NEW) Procedure according to Claim 1, wherein the procedure is indicative of risk of vascular diseases.

36. (NEW) Diagnostic product according to Claim 18, wherein the diagnostic product is indicative of risk of vascular diseases.

REMARKS

The above claims have been amended to remove multiple dependencies.

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Printed Name Shawn Hise

Signature

Respectfully submitted,

HEINRICH WIELAND ET AL.

By their Representatives,

SCHWEGMAN, LUNDBERG, WOESSNER & KLUTH, P.A.
P.O. Box 2938
Minneapolis, MN 55402
(612) 349-9592

Date June 16, 2000 By Ann M. McCrackin

Ann M. McCrackin
Reg. No. 42,858

PROCEDURE AND DIAGNOSTIC PRODUCT FOR THE DETERMINATION OF TRIGLYCERIDE CONTAINED IN LIPOPROTEIN

The present invention relates to a procedure or a diagnostic product for the determination of triglyceride contained in lipoprotein.

Coronary heart disease (CHD) is still the main cause of death in the Western industrial nations. While the importance of cholesterol as a risk factor for coronary heart disease is generally recognized, the assessment of protein-associated triglycerides, in particular of the lipoproteins present in the blood serum, is also taken into consideration in this connection.

As a rule, the division of lipoprotein fractions is carried out, on the basis of differing density, into lipoproteins with very low density ("very low density lipoproteins", abbreviated VLDL below), lipoproteins with low density ("low density lipoproteins", LDL) and lipoproteins with high density ("high density lipoproteins", HDL).

A further specified lipoprotein class is that of the chylomicron (CM).

The lipoproteins can moreover be divided into further subfractions. Among these, particularly the "intermediate density proteins" (IDL) and the "small dense LDL" have great importance for the formation of CHD. The two subfractions of LDL mentioned are particularly triglyceride-rich, so that the LDL triglycerides are more meaningful than the established LDL cholesterol with reference to the CHD risk.

For the diagnosis of vascular diseases, such as coronary heart disease, peripheral arterial occlusive disease and micro- or macroangiopathic changes, the triglyceride content in the individual lipoprotein fractions and the relative amounts contained in the lipoprotein fractions are mutually of importance. For the LDL fraction in particular, it is assumed that a high triglyceride content is associated with coronary heart disease.

Conventional procedures for the determination of triglyceride contained in lipoprotein are essentially based on a two-stage process.

A fractionation step is first carried out in order to separate - as specifically as possible - the respective lipoprotein fractions. A step for the determination of triglyceride in the accordingly separated lipoprotein fractions is then carried out.

Different methods are available for the fractionation step.

The precipitation method is primarily designed for the determination of the triglyceride content in lipoproteins of high density (HDL). The selective precipitation of LDL triglycerides has admittedly been attempted. However, the pure precipitation method proved unsuitable, as considerable amounts of VLDL coprecipitate with the LDL fraction, so that a differentiation of the triglyceride content in the respective lipoproteins is only possible with difficulty (see

R. Siekmeier et al. in Clin. Chim. Acta 177, p. 231 (1988), R. Siekmeier et al. in Clin. Chem. 36, p. 2109-2113 (1990), and M. Nauck et al. in Klin. Lab. 40, p. 167-176 (1994)).

LDL triglycerides were therefore determined in practice by means of sequential ultracentrifugation, according to their density, in the ultracentrifuge, the period up to the obtainment of the LDL fraction amounting to 48 hours, or by a shortened, combined procedure of ultracentrifugation and precipitation.

In the last-mentioned, relatively selective separation, the VLDL fraction is first separated using the ultracentrifuge (time approximately 24 h), and the remaining LDL fraction is then precipitated more or less selectively by suitable agents (Manual of Laboratory Operation, DHEW No. (NIH) 75-628 National Heart and Lung Institute; Lipid Research Clinics Program, Bethesda, MD, USA, p. 1-74 (1979)).

After this, the amount of LDL triglycerides is determined arithmetically from the triglyceride concentration before and after the LDL precipitation.

Electrophoretic separation of the lipoproteins in a suitable carrier matrix, for example an agarose gel, as described in DE 195 20 210 A1, offers a further fractionation method.

General disadvantages of these conventional procedures for the specific determination of triglycerides in lipoprotein fractions result from the fact that the fractionation steps are both labour- and time-intensive. These conventional methods can also be automated poorly or not at all. Without such a fractionation step, however, the diagnostic result based on lipoprotein-associated triglycerides as a risk factor for vascular diseases is virtually unavailable, as only the selective assignment of the triglyceride content to individual or different lipoprotein fractions allows a meaningful risk assessment. In particular if the LDL triglyceride concentration is regarded as particularly meaningful for the prevention of coronary heart diseases, then the routine detection or determination of the LDL triglycerides is especially desirable.

The invention is therefore based on the object of making available a simple, rapid and reliable procedure for the determination of triglyceride contained in lipoprotein, where a selectivity which is as good as possible with respect to the individual lipoprotein fractions, in particular with respect to the diagnostically particularly meaningful LDL triglyceride content, is made possible.

This object is achieved by a procedure for the determination of triglyceride contained in lipoprotein having the following measures:

a) reaction of triglyceride-containing lipoprotein with a non-ionic surface-active agent, which is synthesized from a block copolymer of propylene oxide and ethylene oxide, and

b) carrying-out of a triglyceride determination method.

Further subjects of the present invention consist in a diagnostic product, which is particularly suitable for carrying out this abovementioned procedure, according to Claim 18, and in the use of the abovementioned procedure or of the diagnostic product for the in-vitro diagnosis of vascular disorders according to Claim 34.

According to the invention, it has surprisingly been found that the use of block copolymers synthesized from polypropylene oxide units and polyethylene oxide units, as a particular type of non-ionic surface-active agents, allows an excellent selectivity of the triglyceride determination with respect to only one or certain classes of lipoprotein fractions. A particularly high selectivity owing to the use of the polyoxypropylene/polyoxyethylene block copolymers (abbreviated POP/POE below) is obtained in relation to the LDL lipoprotein fraction, such that the procedure according to the invention is particularly highly suitable for the selective determination of LDL triglyceride. Such a selectivity for the determination of triglyceride from LDL lipoprotein makes the diagnosis particularly meaningful for the area concerned here.

A particular advantage of the invention consists in the fact that the differentiation according to lipoprotein fractions takes place from homogeneous solution. A specific fractionation step, as was necessary according to the conventional procedure described at the outset, is therefore no longer necessary in the procedure according to the invention. In particular, no precipitation step for the separation of specific lipoprotein fractions is necessary, so that the determination of triglyceride can be carried out without a centrifugation step. As furthermore, owing to the use of the specific non-ionic surface-active agent, turbidity of the homogeneous solution can be avoided, it is possible to determine and to quantify the amount of triglyceride from the selectively solubilized lipoprotein fraction in a simple and rapid manner. This makes the procedure according to the invention particularly readily accessible to routine diagnosis as an easily automatable system.

As a basis for these advantageous actions, it is suspected that the use of POP-POE as a non-ionic surface-active agent makes possible a selective solubilization of specific lipoprotein fractions, so that the triglyceride originally associated with this lipoprotein fraction is made accessible and reactive to the determination and detection reagents for triglyceride, whereas other lipoprotein fractions are solubilized less strongly to not at all and thus the triglyceride contained therein is not accessible to determination and quantification. The selectivity in relation to the individual lipoprotein fractions can be adjusted, as desired, by means of the composition of the POP/POE block copolymer. If it is taken into consideration that a block copolymer of this type is synthesized from a

relatively hydrophilic block A having ethylene oxide units and a relatively hydrophobic block B having propylene oxide monomers, by variation of the block units, both within the respective block unit A or B and in the ratio of these units to one another, specific block copolymers can form, which then produce a desired selectivity for the solubilization of a specific lipoprotein or of a group of two lipoprotein classes. Suitable influencing parameters here are the degree of polymerization or the polymerization length within the individual block units A or B and the arrangement and proportioning of the block units relative to the total copolymer. A general overview of block copolymers of propylene oxide and ethylene oxide, from which the materials then suitable for the solubilization of individual lipoprotein fractions can be selected, results from the review articles of I.R. Schmolka in J. Am. Oil Chem. Soc. 54, p. 110 (1977), M.A. Plant in R.D. Karsa (Ed.): "Industrial Applications of Surfactants", The Royal Society of Chemistry, London, p. 318-332 (1986) and K. Kosswig in "Ulmann's Encyclopaedia of Industrial Chemistry", Vol. A 25, p. 747-817, "Surfactants", in particular Chapter 10.1 (1994), where the last-mentioned reference also gives a list of the possible manufacturers.

As the diagnostic meaningfulness as a result of a selective determination of LDL-associated triglyceride is particularly good, in the following the POP/POE block copolymers are described in greater detail which are distinguished by an excellent selectivity for the solubilization of LDL and the making accessible associated therewith of LDL-associated triglyceride to determination and detection reagents.

According to this preferred embodiment of the present invention, the block copolymer consists of a triblock copolymer A-B-A of polyoxyethylene blocks A and a central polyoxypropylene block B. It has emerged that a particularly high selectivity for the determination of LDL triglyceride results when the molecular weight of the POP/POE triblock polymer A-B-A is in the range from 1000 to 8000. The observance of the ratio of the central hydrophobic block constituent B to the terminal hydrophilic block constituents A furthermore has a particularly favourable effect. It was found that the selectivity for the solubilization of LDL triglyceride is particularly favourable if the molecular partial mass of the polyoxypropylene block B with respect to the total triblock copolymer A-B-A is in the range from 75 to 95%, in particular from 85 to 95%. It is assumed that in the case of the observation of the above conditions the hydrophilic/lipophilic balance (HLB) is adjusted such that the structure in the LDL fraction is destabilized, while the structures in the other lipoprotein fractions (HDL, VLDL and CM) remain relatively stable, and thus the triglycerides contained therein are not available or are only available to a relatively small extent for the determination. Consequently, it results from the

abovementioned findings that the selectivity in relation to LDL triglyceride is increased with the hydrophobicity accompanying the increase in the molecular mass fraction of the POP block B.

The amount of the POP/POE block copolymer in a reagent formulated for reaction with a triglyceride lipoprotein-containing sample is suitably in the range from 0.001 to 10% by weight, preferably from 0.01 to 5% by weight and in particular from 0.1 to 1% by weight.

Moreover, it has been found that the selectivity in relation to individual lipoprotein fractions can be increased in the context of the procedure according to the invention by reacting the lipoprotein-containing samples further with agents for the aggregation of lipoprotein fractions. The basis for this selectivity increase by aggregating agents is suspected to lie in the fact that the lipoprotein fractions, which are less strongly solubilized by the appropriately selected POP/POE material, are additionally stabilized by the aggregation.

Examples of suitable agents for the aggregation of lipoprotein fractions include heparin or its salt, phosphotungstic acid or its salt, dextransulphuric acid or its salt, polyethylene glycol, sulphatized cyclodextrin or its salt, sulphatized oligosaccharide or its salt, and mixtures thereof. Examples of cyclodextrin include α-cyclodextrin, β-cyclodextrin and γ-cyclodextrin. Examples of the oligosaccharide include maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose. Salts which can be used are, for example, the sodium, potassium, lithium, ammonium and magnesium salts.

A preferred aggregating agent is cyclodextrin or a cyclodextrin derivative. In particular when using sulphatized α-cyclodextrin, it has advantageously been shown that the selectivity is improved with respect to LDL-associated triglyceride.

A further preferred aggregating agent is dextran-sulphuric acid or its salt dextran sulphate.

Again, with respect to the preferred selectivity according to the invention in relation to LDL triglyceride, it was found that a combination of sulphatized α-cyclodextrin with dextran sulphate in particular had an increased action. For assistance or stabilization of the aggregation of the lipoprotein fractions which are not specifically to be solubilized by the specific POP/POE surface-active agent, in addition to the aggregating agent, salts of divalent metal ions should furthermore be employed. Examples of suitable divalent metal ions are magnesium, manganese, calcium, nickel and cobalt, magnesium being preferred.

The amounts of the aggregating agents, or the salts of divalent metal ions, optionally to be employed can be adapted to the respective case taking into account the desired selectivity with respect to individual lipoprotein fractions and

the nature of the aggregating agent. The preferred lower limit content is in this case fixed by a desired and detectable stabilization effect, while the preferred upper limit content is fixed by the avoidance of appearance of turbidity and in particular the avoidance of precipitation, which would prevent a direct triglyceride determination from homogeneous solution.

Suitable amounts of the abovementioned constituents contained in an appropriately formulated reagent lie in the following ranges: 0.02 to 10 mM heparin having a molecular weight of 5000 to 20,000 or its salt, 0.1 to 10 mM phosphotungstic acid having a molecular weight of 4000 to 8000 or its salt, 0.01 to 5 mM dextransulphuric acid having a molecular weight of 10,000 to 500,000 or 0.1 to 20 mM dextransulphuric acid having a molecular weight of 1000 to 10,000 or its salts, 0.3 to 100 mM polyethylene glycol (PEG) having a molecular weight of 4000 to 25,000, 0.1 to 50 mM sulphatized cyclodextrin having a molecular weight of 1000 to 3000 or its salt, 0.1 to 50 mM sulphatized oligosaccharide having a molecular weight of 400 to 3000 or its salt, and mixtures thereof. 0.03 to 1 mM heparin having a molecular weight of 14,000 to 16,000 or its salt, 0.1 to 3 mM phosphotungstic acid having a molecular weight of 5000 to 7000 or its salt, 0.01 to 5 mM dextran sulphate having a molecular weight of 150,000 to 250,000 or its salt, 0.1 to 10 mM dextran sulphuric acid having a molecular weight of 1000 to 5000 or its salt, 1.0 to 50 mM PEG having a molecular weight of 5000 to 22,000, 0.1 to 10 mM sulphatized cyclodextrin having a molecular weight of 1000 to 2000 or its salt, 0.1 to 10 mM sulphatized oligosaccharide having a molecular weight of 400 to 2000 or its salt, and mixtures thereof are preferred.

The concentration of the salt of divalent metal ions is suitably 0.1 to 50 mM, preferably 1 to 5 mM.

The further measure b) of the procedure according to the invention consists in the carrying-out of a triglyceride determination method. For this, determination methods which are known per se and, for example, the determination methods employed in the conventional lipoprotein triglyceride determination procedures mentioned at the outset can be employed. In this case, the use of the enzymatic determination methods customarily carried out has an advantageous effect for the concept according to the invention, since the enzymes employed for this are able, on the one hand, to reach the triglyceride in the specifically destabilized or solubilized lipoprotein fractions and thus to react (which primarily relates to the enzymatic cleavage of triglyceride with formation of glycerol), whereas the lipoprotein fractions not solubilized as a matter of priority and lipoprotein fractions optionally additionally stabilized by aggregating agents protect the triglyceride associated there from the enzymatic reaction.

The enzymatic cleavage is expediently carried out with the aid of lipase or an esterase. The glycerol released thereby can be determined and quantified by enzymatic photometric tests and in particular by means of colour detection reactions. An overview of commercially obtainable tests for carrying out the triglyceride determination is given by A. Bruckner and M. Moret in J. Clin. Chem. Clin. Biochem., Vol. 21, p. 97-106 (1983).

According to the invention, a determination method has proved particularly sensitive which consists in determining the glycerol released as described beforehand by enzymatic reaction using the enzymes glycerokinase and glycerol 3-phosphate dehydrogenase, by means of which a reduced acceptor of reduction/oxidation equivalents, such as NAD or FMN, is formed, which for its part is then determined by a detection reaction.

As a sensitive detection reaction, the carrying-out of a colour reaction is recommended, in which, by means of an electron coupler, a dye is reduced by the reduced acceptor or reduction/oxidation equivalents such as NADH or $FMNH_2$, the reduced form of which dye can be determined photometrically by means of the corresponding absorption wavelength. Suitable electron couplers are, for example, the enzyme diaphorase or the synthetic phenacin methosulphate. Examples of dyes are tetrazolium salts, such as Tetrazolium Blue, Nitro Blue Tetrazolium (NBT), Tetrazolium Violet, Tetrazolium Purple and 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride (INT). These dyes react with formazan formation to give dyes which can be photometrically determined and quantified at the appropriate absorption wavelength, in the case of NBT or INT, for example, at 570 nm.

Other examples, in particular with respect to a high sensitivity, include fluorometric and luminometric determinations.

A further sensitivity increase in connection with the carrying-out of the triglyceride determination method is obtained by including the enzymatic reaction with the released glycerol in addition to the use of the enzymes triosephosphate isomerase and glyceraldehyde 3-phosphate dehydrogenase. The sensitivity increase results in that, per molecule of glycerol released, not only one, but two molecules of reduced reduction/oxidation equivalent are produced. Per glycerol molecule released, two molecules of reduced reduction/oxidation equivalents, such as NADH or FADH, are accordingly available, which consequently also doubles the detection sensitivity.

A particular advantage of the invention results from the fact that both the reaction of triglyceride-containing lipoprotein with the specific POP/POE surface-active agent (measure a)) and the carrying-out of the triglyceride determination method (measure b)) can be allowed to proceed simultaneously.

Owing to this, the two-stage procedure which is conventionally necessary is reduced to a one-stage procedure. Furthermore, labour- and time-consuming fractionation steps are no longer necessary; the incubation according to measure a) and the triglyceride determination according to measure b) can be carried out simultaneously in one batch or at least overlapping in terms of time. If, according to the preferred embodiment of the present invention for increasing the selectivity, an agent for the aggregation of lipoproteins and, if appropriate, furthermore the salt of divalent metal ions is used, however, it has proved expedient first to incubate these constituents with the sample to be determined briefly (for example for a couple of minutes), and only then to add the specific POP/POE surface-active agent and the reagents for the triglyceride determination to this batch. After a further incubation time of a few minutes, the appropriate detection, for example the described photometric determination, can then be carried out.

The incubation for selectively making accessible or releasing triglyceride from specific lipoprotein fractions and the simultaneous or subsequent carrying-out of the triglyceride determination method takes place in a suitable buffer system, which is preferably buffered to a pH range of 5 to 9 and in particular of approximately 6.5 to 9. A glycylglycine buffer or a tris buffer, for example, in a concentration of 5 to 500 mM is suitable. A donor of energy-rich phosphate groups, such as ATP (e.g. 0.1 mM to 50 mM ATP), a calcium ion chelator such as EDTA (e.g. 0 to 5 mM EDTA) and magnesium salt such as $MgCl_2$ (e.g. 1 mM to 50 mM) are moreover suitably employed for the enzymatic reactions.

For the practical implementation of the procedure according to the invention, the triglyceride-associated, lipoprotein-containing biological sample, which as a rule consists of a blood sample (serum or plasma) or of a urine sample, is mixed with the reagent containing the constituents described beforehand at a suitable dilution, which is approximately in the range from 0.1:100 to 10:100 and in particular in the range from 0.5:100 to 2:100. In the case of the preferred use of the aggregating agent and, if appropriate, of the divalent metal ions, a dilution mixture is first prepared in the manner described beforehand using the reagent containing these constituents and briefly incubated, after which the reagent is then added with the agents described for measures a) and b).

The invention furthermore makes available a diagnostic product which is particularly suitable for carrying out the procedure according to the invention, which - in at least one reagent of the diagnostic product - as constituent a) (corresponding to procedure measure a)) includes the specific surface-active agent described beforehand and, as constituent b) (corresponding to procedure measure b)) the described agent(s) for the determination of triglyceride. With respect to the

description of constituent a) and of constituent b), reference can be made to the above description of the corresponding procedure measures.

In order that the incubation with the surface-active agent and the incubation for the triglyceride determination proceed simultaneously in an advantageous manner, the diagnostic product is preferably designed as a kit, and the constituents a) and b) are in this case combined in one reagent or two reagents of the diagnostic kit.

In a preferred embodiment of the diagnostic product, this furthermore contains as a further constituent agents for the aggregation of lipoprotein fractions and, if appropriate, a salt of divalent metal ions. Reference can also be made in this respect to the above description. The agent(s) for the aggregation, and, if appropriate, the salt of divalent metal ions is or are preferably contained in a reagent of the diagnostic kit which is different from the reagent comprising the abovementioned constituents a) and b). This allows the above-described, advantageous preference of the incubation of the sample to be determined with the stabilizing aggregating agents, before the reaction with the specific surface-active agent and the optionally simultaneous carrying-out of the triglyceride determination follows.

The present invention is distinguished by a high selectivity in relation to lipoprotein fractions in homogeneous, liquid phase. This applies in particular to the selective determination of LDL triglyceride under the conditions described above.

On comparison with conventional determination procedures, it was found that the results obtained by means of the invention correlate very well with those of the prior art. However, according to the invention a small amount of the sample to be investigated suffices, and the specific lipoprotein-associated triglyceride can be determined in a short time of even a few minutes. The determination can furthermore be carried out directly from the homogeneous phase, so that two-stage processes which include complicated fractionation steps are no longer necessary.

The present invention is therefore excellently suited for simple and reliable routine diagnosis and ought to be accessible to automation. A diagnostic possibility which suggests itself is primarily the use of the procedure or diagnostic product according to the invention for the in-vitro diagnosis or determination of the risk of vascular diseases.

In this connection, the determination of LDL triglycerides as a universal risk indicator for coronary heart disease, furthermore for diabetic macro- and microangiopathy and as an indicator for LDL of atypical composition (type III hyperlipoproteinaemia according to Fredrickson) can be mentioned in particular.

The invention is illustrated in greater detail below by means of the following examples.

Example 1

For the selective determination of LDL triglyceride, a reagent having the following constituents was first formulated.

POP/POE triblock copolymer, molecular weight 4500, POP/POE proportion 90% by weight: 0.1% by weight

Lipase: 10 kU/l

Glycerokinase: 4.8 kU/l

Glycerol 3-phosphate dehydrogenase: 48 kU/l

Triosephosphate isomerase: 300 kU/l

Glyceraldehyde 3-phosphate dehydrogenase: 24 kU/l

Diaphorase: 4.8 kU/l

ATP: 5 mM

NAD: 5 mM

EDTA: 0.5 mM

4-NBT: 3 mM

Glycylglycine buffer (pH 7.5): 0.2 M, made up to 100% by weight.

4 µl of a serum sample were added to 400 µl of this reagent and incubated at 37°C for 5 min. The dye formed up to this time was determined photometrically at 570 nm.

For quantification, a standardization measurement was additionally carried out. For this, a defined amount of LDL triglyceride isolated by ultracentrifugation was initially added (5 g/l) and diluted to a dilution of 1:10 with isotonic saline solution (0.9% by weight) in a fixed dilution series. The respective dilutions were measured analogously to the procedure described beforehand. A linear standard curve resulted within the prepared dilution series.

Furthermore, for the specific quantification of LDL triglyceride from the serum sample, the total triglyceride was determined using a commercially obtainable serum triglyceride test.

In a comparison with conventional, two-stage determination procedures such as ultracentrifugation and the precipitation technique, adequately corresponding values resulted by means of the procedure according to the invention.

Example 2

Example 1 was repeated in the same manner with the assumption that, instead of the POP/POE block copolymer employed there, one having a molecular

partial mass of the POP block with respect to the total block copolymer of 70% by weight was used.

The result obtained showed that the reactivity of the triglyceride determination with respect to the specific LDL species was admittedly just as good as in Example 1, but that an - even if slight - reactivity to other lipoprotein species was to be observed. Consequently, although the selectivity with respect to the LDL triglyceride determination was still practically acceptable, it was somewhat poorer than in Example 1.

Example 3

A first reagent having the following constituents was initially formulated:

Sulphatized α-cyclodextrin: 0.5 mM

Dextran sulphate (molecular weight 200,000): 1 mM

$MgCl_2$: 2.5 mM

Glycylglycine buffer (pH 7.2): 0.2 M, made up to 100% by weight.

For carrying out the specific LDL triglyceride determination, 4 µl of the serum sample were added to 300 µl of this reagent, and the mixture was incubated at 37°C for 5 min. 100 µl of a reagent analogous to Example 1, in which the concentration of the reagent constituents apart from those of the buffer was four time higher, were added and the mixture was again incubated for 5 min. The measurement of the LDL triglyceride, the comparison with the standard curve and the total serum triglyceride measurement were carried out in the same manner as described in Example 1.

The results obtained showed an even better agreement with the conventional, two-stage triglyceride determination procedures and thus an even better selectivity of the LDL triglyceride determination.

Patent claims

1. Procedure for the determination of triglyceride contained in lipoprotein having the following measures:

a) reaction of triglyceride-containing lipoprotein with a non-ionic surface-active agent, which is synthesized from a block copolymer of propylene oxide and ethylene oxide, and

b) carrying-out of a triglyceride determination method.

2. Procedure according to Claim 1, characterized in that it is used for the selective determination of LDL triglyceride.

3. Procedure according to Claim 1 or 2, characterized in that it is carried out in homogeneous solution.

4. Procedure according to one of the preceding claims, characterized in that the block copolymer used is an A-B-A triblock copolymer of polyoxyethylene blocks A and central polyoxypropylene block B.

5. Procedure according to Claim 4, characterized in that, for the selective determination of LDL triglyceride, the molecular weight of the polyoxypropylene/polyoxyethylene triblock copolymer A-B-A is in the range from 1000 to 8000.

6. Procedure according to Claim 5, characterized in that the molecular partial mass of the polyoxypropylene block B with respect to the total triblock copolymer A-B-A is in the range from 75 to 95% by weight.

7. Procedure according to one of the preceding claims, characterized in that the reaction according to measure a) and the triglyceride determination according to measure b) are carried out simultaneously.

8. Procedure according to one of the preceding claims, characterized in that the triglyceride-containing lipoproteins are furthermore reacted with agents for the aggregation of lipoprotein fractions.

9. Procedure according to Claim 8, characterized in that the agent used for the aggregation of lipoprotein fractions is cyclodextrin or cyclodextrin derivative.

10. Procedure according to Claim 9, characterized in that sulphatized α-cyclodextrin is used.

11. Procedure according to one of Claims 8 to 10, characterized in that - if appropriate additionally - dextransulphuric acid or its salt is used as an agent for the aggregation of lipoprotein fractions.

12. Procedure according to one of Claims 8 to 11, characterized in that the reaction with the aggregating agent is carried out in the presence of divalent metal ions.

13. Procedure according to one of Claims 8 to 12, characterized in that the reaction with the aggregating agent is carried out before measures a) and b).

14. Procedure according to one of the preceding claims, characterized in that the determination of triglyceride according to measure b) includes the enzymatic cleavage of triglyceride and the determination of the glycerol released thereby.

15. Procedure according to Claim 14, characterized in that the enzymatic cleavage is carried out with the aid of lipase or an esterase.

16. Procedure according to Claim 14 or 15, characterized in that the released glycerol is determined by enzymatic reaction with the enzymes glycerokinase and glycerol 3-phosphate dehydrogenase, by means of which a reduced acceptor of reduction/oxidation equivalents is formed, which is determined by a detection reaction.

17. Procedure according to Claim 16, characterized in that the enzymes triosephosphate isomerase and glyceraldehyde 3-phosphate dehydrogenase are furthermore added to the enzymatic reaction.

18. Diagnostic product for the determination of triglyceride contained in lipoprotein having the following constituents:

a) a non-ionic surface-active agent which is synthesized from a block copolymer of propylene oxide and ethylene oxide, and

b) agents for the determination of triglyceride.

19. Diagnostic product according to Claim 18, characterized in that it is used for the selective determination of LDL triglyceride.

20. Diagnostic product according to Claim 18 or 19, characterized in that as block copolymer constituent a) it contains an A-B-A triblock copolymer of polyoxyethylene blocks A and central polyoxypropylene block B.

21. Diagnostic product according to Claim 20, characterized in that, for the selective determination of LDL triglyceride, the molecular weight of the polyoxypropylene/polyoxyethylene triblock copolymer A-B-A is in the range from 1000 to 8000.

22. Diagnostic product according to Claim 21, characterized in that the molecular partial mass of the polyoxypropylene block B with respect to the total triblock copolymer A-B-A is in the range from 75 to 95% by weight.

23. Diagnostic product according to one of Claims 18 to 22, characterized in that the constituents a) and b) are combined in a reagent of a diagnostic kit.

24. Diagnostic product according to one of Claims 18 to 23, characterized in that, as a further constituent, it contains agents for the aggregation of lipoprotein fractions.

25. Diagnostic product according to Claim 24, characterized in that, as agents for the aggregation of lipoprotein fractions, cyclodextrin or cyclodextrin derivative is present.

26. Diagnostic product according to Claim 25, characterized in that sulphatized α-cyclodextrin is present.

27. Diagnostic product according to one of Claims 24 to 26, characterized in that - if appropriate additionally - dextransulphuric acid or its salt is present as an agent for the aggregation of lipoprotein fractions.

28. Diagnostic product according to one of Claims 24 to 27, characterized in that, in addition to the agent for the aggregation of lipoprotein fractions, divalent metal ions are present.

29. Diagnostic product according to one of Claims 24 to 28, characterized in that the constituent containing the aggregating agent(s) and optionally the divalent metal ions is present in a reagent of a diagnostic kit different from the reagent containing the constituents a) and b).

30. Diagnostic product according to one of Claims 18 to 29, characterized in that constituent b) includes an enzyme for the cleavage of triglyceride and customary agents for the determination of glycerol.

31. Diagnostic product according to Claim 30, characterized in that the enzyme for the cleavage of triglyceride is lipase or an esterase.

32. Diagnostic product according to Claim 30 or 31, characterized in that the agent for the determination of cleaved glycerol includes the enzymes glycerokinase and glycerol 3-phosphate dehydrogenase, and a reduced acceptor of reduction/oxidation equivalents.

33. Diagnostic product according to Claim 32, characterized in that the agent for the determination of glycerol furthermore includes the enzymes triosephosphate isomerase and glyceraldehyde 3-phosphate dehydrogenase.

34. Use of a procedure according to one of Claims 1 to 17 or of a diagnostic product according to one of Claims 18 to 33 for the in-vitro diagnosis or determination of risk of vascular diseases.

Abstract

A procedure is described for the determination of triglyceride contained in lipoprotein with the measures that triglyceride-containing lipoprotein is reacted with a non-ionic surface-active agent which is synthesized from a block copolymer of propylene oxide and ethylene oxide, and that a triglyceride determination method is carried out. In a diagnostic product, the agents for the abovementioned procedural steps are combined as constituents. The procedure and the diagnostic product are particularly suitable for the in-vitro diagnosis of vascular disorders, in particular in the detection of coronary heart disease.

SCHWEGMAN ■ LUNDBERG ■ WOESSNER ■ KLUTH

United States Patent Application

COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor I hereby declare that: my residence, post office address and citizenship are as stated below next to my name; that

I verily believe I am the original, first and joint inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled: **PROCEDURE AND DIAGNOSTIC PRODUCT FOR THE DETERMINATION OF TRIGLYCERIDE CONTAINED IN LIPOPROTEIN**.

The specification of which is attached hereto.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with 37 C.F.R. § 1.56 (attached hereto). I also acknowledge my duty to disclose all information known to be material to patentability which became available between a filing date of a prior application and the national or PCT international filing date in the event this is a Continuation-In-Part application in accordance with 37 C.F.R. §1.63(e).

I hereby claim foreign priority benefits under 35 U.S.C. §119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on the basis of which priority is claimed:

Foreign application(s), if any, claiming priority under 35 U.S.C. § 119:

Application Number	Country	Day/Month/Year Filed
19756255.8	Germany	17/12/1997

I hereby claim the benefit under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below:

No such claim for priority is being made at this time.

I hereby claim the benefit under 35 U.S.C. § 120 or 365(c) of any United States and PCT international application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose material information as defined in 37 C.F.R. § 1.56(a) which became available between the filing date of the prior application and the national or PCT international filing date of this application:

Application Number	Filing Date	Status
PCT/EP98/08253	December 16, 1998	Pending

I hereby appoint the following attorney(s) and/or patent agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected herewith:

Anglin, J. Michael	Reg. No. 24,916	Jurkovich, Patti J.	Reg. No. 44,813	Nelson, Albin J.	Reg. No. 28,650
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Billion, Richard E.	Reg. No. 32,836	Kaufmann, John D.	Reg. No. 24,017	Oh, Allen J.	Reg. No. 42,047
Black, David W.	Reg. No. 42,331	Klima-Silberg, Catherine I.	Reg. No. 40,052	Padys, Danny J.	Reg. No. 35,635
Brennan, Leoniede M.	Reg. No. 35,832	Kluth, Daniel J.	Reg. No. 32,146	Parker, J. Kevin	Reg. No. 33,024
Brennan, Thomas F.	Reg. No. 35,075	Lacy, Rodney L.	Reg. No. 41,136	Perdok, Monique M.	Reg. No. 42,989
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Chu, Dinh C.P.	Reg. No. 41,676	LeMoine, Dana B.	Reg. No. 40,062	Schumm, Sherry W.	Reg. No. 39,422
Clark, Barbara J.	Reg. No. 38,107	Lundberg, Steven W.	Reg. No. 30,568	Schwegman, Micheal L.	Reg. No. 25,816
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Drake, Eduardo E.	Reg. No. 40,594	Maeyaert, Paul L.	Reg. No. 40,076	Speier, Gary J.	Reg. No. 45,458
Embretson, Janet E.	Reg. No. 39,665	Maki, Peter C.	Reg. No. 42,832	Steffey, Charles E.	Reg. No. 25,179
Fordenbacher, Paul J.	Reg. No. 42,546	Malen, Peter L.	Reg. No. 44,894	Terry, Kathleen R.	Reg. No. 31,884
Forrest, Bradley A.	Reg. No. 30,837	Mates, Robert E.	Reg. No. 35,271	Tong, Viet V.	Reg. No. 45,416
Gamon, Owen J.	Reg. No. 36,143	McCrackin, Ann M.	Reg. No. 42,858	Viksnins, Ann S.	Reg. No. 37,748
Harris, Robert J.	Reg. No. 37,346	Nama, Kash	Reg. No. 44,255	Woessner, Warren D.	Reg. No. 30,440
Huebsch, Joseph C.	Reg. No. 42,673				

I hereby authorize them to act and rely on instructions from and communicate directly with the person/assignee/attorney/firm/organization/who/which first sends/sent this case to them and by whom/which I hereby declare that I have consented after full disclosure to be represented unless/until I instruct Schwegman, Lundberg, Woessner & Kluth, P.A. to the contrary.

Please direct all correspondence in this case to **Schwegman, Lundberg, Woessner & Kluth, P.A.** at the address indicated below:

P.O. Box 2938, Minneapolis, MN 55402
Telephone No. (612)373-6900

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full Name of joint inventor number 1 : **Heinrich Wieland**
Citizenship: **Germany** Residence: **St. Peter, Germany**
Post Office Address: In der Wiehre 13,
D-79271
St. Peter
Germany

Signature: _____ Date: _____
Heinrich Wieland

Full Name of joint inventor number 2 : **Matthias Nauck**
Citizenship: **Germany** Residence: **Freiburg i. Br., Germany**
Post Office Address: Kirchenmattenweg 30
D-79110
Freiburg i. Br.
Germany

Signature: _____ Date: _____
Matthais Nauck

§ 1.56 Duty to disclose information material to patentability.

(a) A patent by its very nature is affected with a public interest. The public interest is best served, and the most effective patent examination occurs when, at the time an application is being examined, the Office is aware of and evaluates the teachings of all information material to patentability. Each individual associated with the filing and prosecution of a patent application has a duty of candor and good faith in dealing with the Office, which includes a duty to disclose to the Office all information known to that individual to be material to patentability as defined in this section. The duty to disclose information exists with respect to each pending claim until the claim is canceled or withdrawn from consideration, or the application becomes abandoned. Information material to the patentability of a claim that is canceled or withdrawn from consideration need not be submitted if the information is not material to the patentability of any claim remaining under consideration in the application. There is no duty to submit information which is not material to the patentability of any existing claim. The duty to disclose all information known to be material to patentability is deemed to be satisfied if all information known to be material to patentability of any claim issued in a patent was cited by the Office or submitted to the Office in the manner prescribed by §§ 1.97(b)-(d) and 1.98. However, no patent will be granted on an application in connection with which fraud on the Office was practiced or attempted or the duty of disclosure was violated through bad faith or intentional misconduct. The Office encourages applicants to carefully examine:

(1) prior art cited in search reports of a foreign patent office in a counterpart application, and

(2) the closest information over which individuals associated with the filing or prosecution of a patent application believe any pending claim patentably defines, to make sure that any material information contained therein is disclosed to the Office.

(b) Under this section, information is material to patentability when it is not cumulative to information already of record or being made of record in the application, and

(1) It establishes, by itself or in combination with other information, a prima facie case of unpatentability of a claim; or

(2) It refutes, or is inconsistent with, a position the applicant takes in:

(i) Opposing an argument of unpatentability relied on by the Office, or

(ii) Asserting an argument of patentability.

A prima facie case of unpatentability is established when the information compels a conclusion that a claim is unpatentable under the preponderance of evidence, burden-of-proof standard, giving each term in the claim its broadest reasonable construction consistent with the specification, and before any consideration is given to evidence which may be submitted in an attempt to establish a contrary conclusion of patentability.

(c) Individuals associated with the filing or prosecution of a patent application within the meaning of this section are:

(1) Each inventor named in the application:

(2) Each attorney or agent who prepares or prosecutes the application; and

(3) Every other person who is substantively involved in the preparation or prosecution of the application and who is associated with the inventor, with the assignee or with anyone to whom there is an obligation to assign the application.

(d) Individuals other than the attorney, agent or inventor may comply with this section by disclosing information to the attorney, agent, or inventor.